

Effects of Retinoic Acid on Wingless (WNT)  
Signaling in the Hair Follicles of Mice

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## **Abstract**

Many cancers in the skin arise from aberrant hair follicle stem cells. WNT (wingless related mouse mammary tumor virus) signaling is important in regulating these stem cells, as well as in initiating the growth phase of the hair cycle (anagen). Follicular localization sites (including hair follicle stem cells) of several WNT signaling molecules are similar to those of synthesis enzymes of retinoic acid (RA), a vitamin A metabolite. As vitamin A and metabolites (retinoids/RA) are essential for growth, vision, reproduction, cell proliferation, differentiation, and apoptosis, exogenous retinoid treatments are currently used for various human skin diseases. However, the specific effects of endogenous retinoids (particularly RA) on hair follicles remain uncertain. To gain a better understanding of how RA interacts with WNT signaling, we performed 3 preliminary studies. First, we analyzed the localization of  $\beta$ -catenin in hair follicles of mice, as nuclear  $\beta$ -catenin marks sites of WNT signaling. Mice were wax-stripped to synchronize the hair cycle and tissues were collected. Immunohistochemistry (IHC) determined that nuclear  $\beta$ -catenin peaked 6 days after wax stripping, when hair was in mid-anagen. Second, we determined a drug and dose that topically inhibited RA synthesis, and whether this inhibition affected WNT signaling. Additional mice were wax-stripped, and 6 days later, 4 doses of citral or disulfiram were applied to this area. IHC determined that the highest dose of each drug best inhibited RAR- $\beta$ , a RA target gene, and citral provided stronger inhibition overall. However, citral did not completely inhibit RA synthesis, indicating that further studies should be conducted using higher doses in order to determine the optimal amount to accomplish this. IHC analysis on the dorsal skin tissue from these mice also found that when RA is inhibited, WNT signaling is active. Third, we determined the easiest and most cost effective manner to measure WNT signaling. Two additional mice were wax stripped, and 6 days later, dorsal skin was

collected. IHC determined that the  $\beta$ -galactosidase antibody provided an easier way to determine active WNT signaling when compared to nuclear  $\beta$ -catenin localization. A large-scale experiment, analyzing the effects of topically applied RA inhibitors on WNT signaling, can be performed once the optimal dose is determined. Interactions between RA and WNT signaling may impact the development of treatments for hair diseases and skin cancer.

## **Introduction**

Vitamin A (retinol) and its active derivatives (retinoids/retinoic acid) are essential for maintenance of several tissues, including the skin and hair (Wolbach and Howe, 1925).

While deficiency modifies stem cell function, re-administration of vitamin A (Wolbach and Howe, 1933) causes the modified cells to undergo apoptosis. These observations demonstrate the importance of maintaining the recommended amounts of vitamin A in cells and have led to the use of exogenous retinoid treatments for various human skin diseases (Jones, 2005). However, the specific effects of endogenous retinoids on hair follicles remain uncertain. Hair follicles are constantly cycling from anagen (growing phase) through catagen (regression), and into telogen (resting) (Stenn and Paus, 2001; Alonso and Fuchs, 2006).

Wingless related mouse mammary tumor virus (WNT) signaling is essential for initiation of the growing phase of the hair cycle (Lo Celso *et al.*, 2004; Shimizu and Morgan, 2004).

Previous work conducted in this laboratory demonstrated the detailed localization patterns of retinoic acid (RA) biosynthesis enzymes and signaling components throughout the murine hair cycle (Everts *et al.*, 2007). Considering that these sites of expression are similar to those of several WNT signaling molecules (Reddy *et al.*, 2001) and RA interacts with WNT signaling in other tissues and cell lines (DasGupta and Fuchs, 1999; Huelsken *et al.*, 2001; Reddy *et al.*, 2001; Lo Celso *et al.*, 2003; Shimizu and Morgan, 2004), RA may also interact

with WNT signaling in the hair follicle. Therefore, the overall objective of my thesis is to study the effects of RA on WNT signaling in the hair follicles of mice. My *hypothesis* is that the presence of retinoic acid (RA) will inhibit WNT signaling. To gain a better understanding of how RA interacts with WNT signaling, we performed three preliminary studies: 1) Determined where and when during the hair cycle nuclear  $\beta$ -catenin co-localizes with RA synthesis; 2) Determined a drug and dose that inhibits RA synthesis in the skin in order to determine if RA inhibits WNT signaling; 3) Determined the easiest and most cost effective manner to measure WNT signaling.

## **Literature Review**

Hairs are mini-organs that are maintained through a cyclic process that includes periodic stem-cell dependent regeneration of hair follicles throughout adult life (reviewed in Stenn and Paus, 2001; Alonso and Fuchs, 2006). The hair follicle (HF) consists of a permanent portion from the bulge upward, and a lower portion that cycles between phases of rapid growth (anagen), regression (catagen), and rest (telogen) in order to produce new hairs (**Figure 1**). Throughout anagen (**Figure 2**), an entire hair shaft from tip to root is produced, while two thirds of the cycling portion of the HF undergoes apoptosis during catagen. Telogen consists of a resting period in which the stem cells of the follicles prepare for a signal that begins the growth of another shaft, or the next anagen phase. Studies have been conducted on these three major phases of the HF cycle in humans (Kligman, 1959), as well as in many mouse strains (Orwin *et al*, 1967; Panteleyev *et al*, 1998, 1999). A comprehensive guide on murine (mouse) HF cycling was established by Paus and colleagues in 2001 (Muller-Rover *et al*, 2001). This guide summarizes criteria for recognizing distinct stages of the murine hair cycle based on previous staging guides (Paus *et al* 1999) and

Figure 1: Schematic of the cycling hair follicle

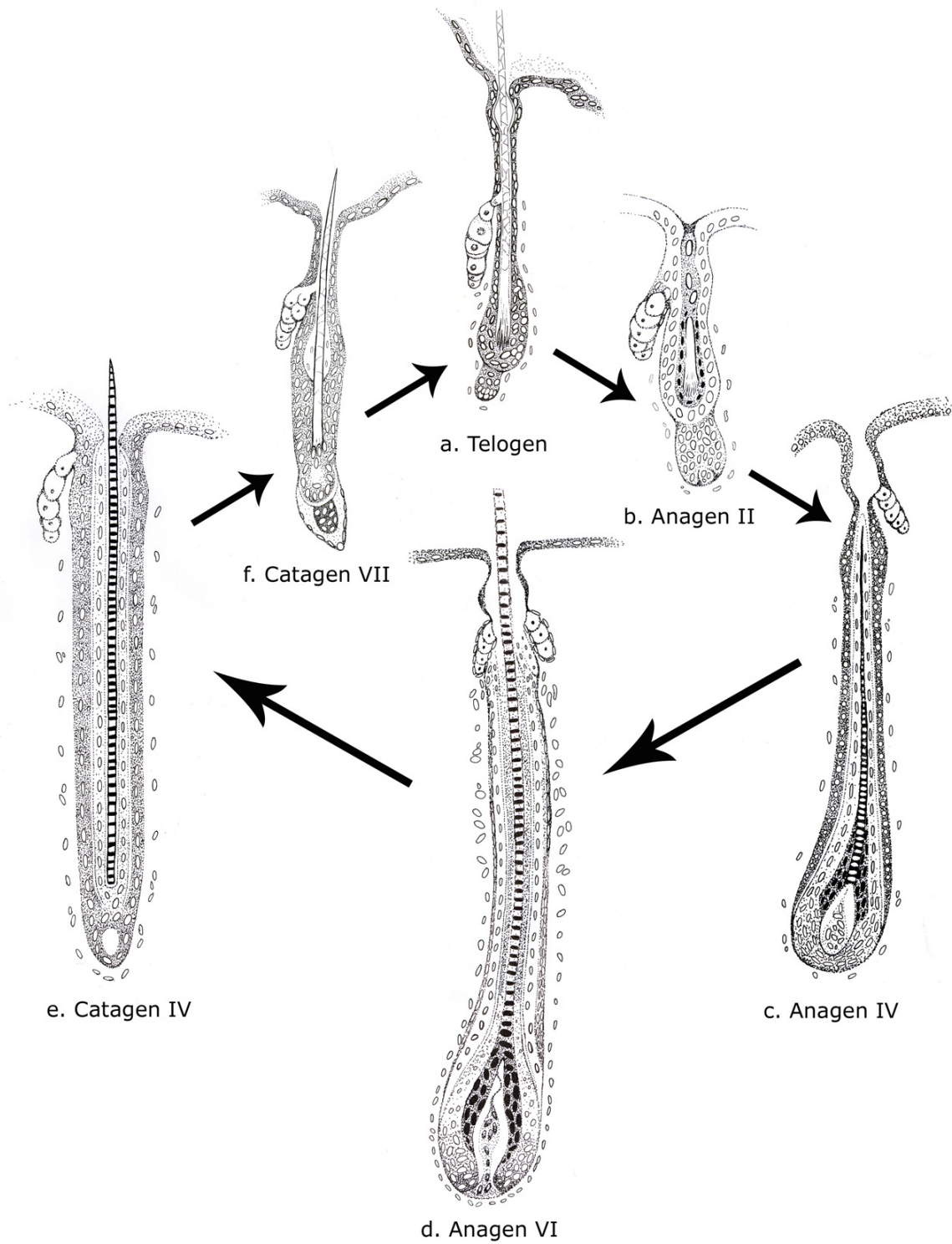
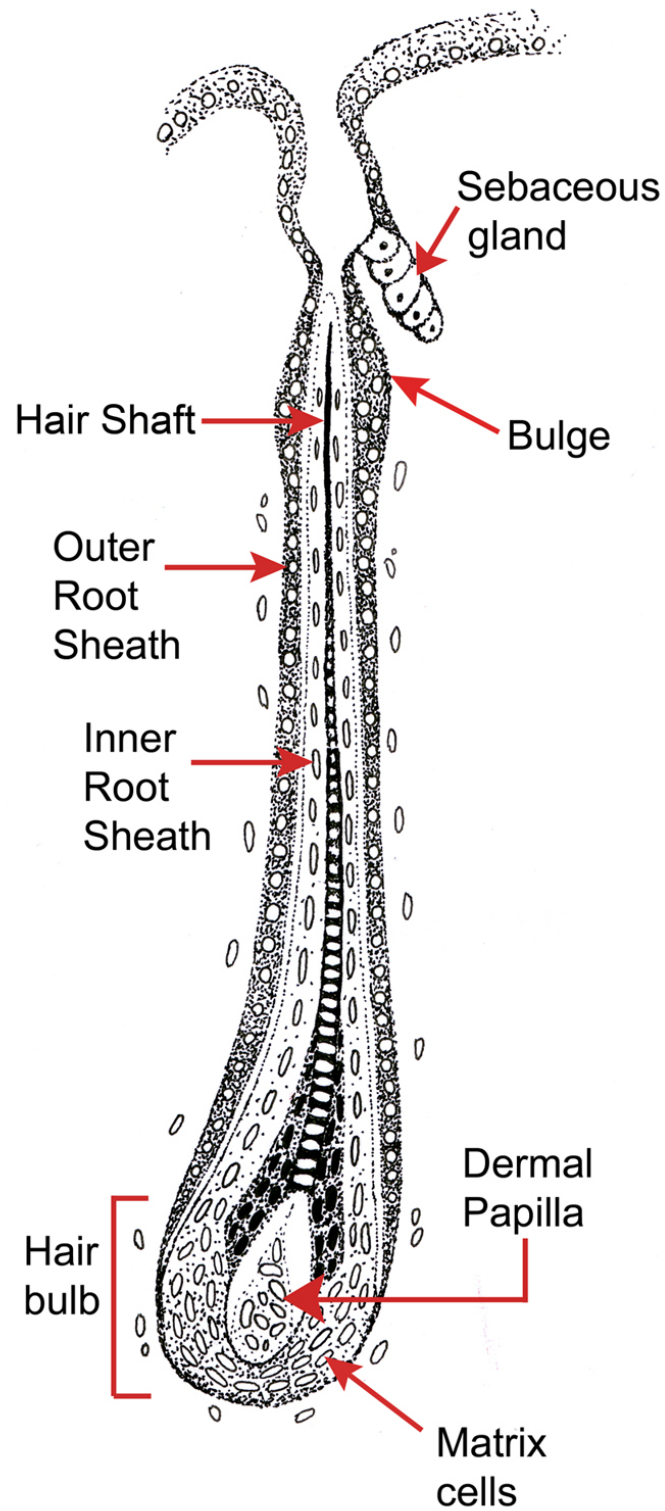


Figure 2: Description of an anagen follicle





fundamental histological and ultrastructural studies on murine cycling hair follicles (Wolbach, 1951; Chase *et al*, 1951). These staging guides are also relevant for human hair, as equivalent critical follicle transformations occur in mice and humans (Kligman, 1959). The guides indicate that there are six major and three substages of anagen, eight stages of catagen, and the resting phase of telogen (Muller-Rover *et al*, 2001). As HF are sometimes inhibited from entering anagen after telogen during cycling, two functional phases of telogen exist: competent and refractory (Plikus *et al*, 2008), which will be discussed later in further detail.

The hair cycle is regulated through growth factors of the specialized dermal cells (that make up the dermal papilla) interacting with the epithelial stem cells, the epithelial matrix, and pre-cortical cells (reviewed in Stenn and Paus, 2001). The two possible resting phases of telogen determine whether a HF will transition from telogen to anagen. Competent telogen allows for anagen re-entry and regeneration of a new hair, while refractory telogen arrests the follicle in this stage (Plikus *et al*, 2008). Re-entry into anagen is required for growth of a new hair, and ultimately involves activation of epithelial stem cells (reviewed in Stenn and Paus, 2001; Alonso and Fuchs, 2006). These stem cells are thought to reside in the base of the upper half of the HF, in an area known as the bulge (Cotsarelis *et al*, 1990; Oshima *et al*, 2001; reviewed in Alonso and Fuchs, 2003). The bulge is located within the outer root sheath (ORS) just below the opening of the sebaceous gland, and serves as the attachment site for the arrector pili muscle (Montagna, 1962). As this area marks the lower end of the permanent portion of the HF, the matrix cells (keratinocytes) below it are thought to begin cycling through the phases of the hair cycle according to the “bulge activation hypothesis” (Cotsarelis, *et al*, 1990; reviewed in Alonso and Fuchs, 2003). The dermal papilla resides in the lower portion of the HF just below the epithelium, and undergoes hair-cycle dependent

changes in its volume, appearance, and activity (Montagna, 1962). The “bulge activation hypothesis” suggests that bulge stem cells proliferate and migrate downward to ultimately surround the dermal papilla and form the bulb, only after receiving signals from specialized HF mesenchymal stem cells. As bulge cells have been shown to divide before re-growth of the HF, this hypothesis is supported (Wilson *et al.*, 1994). The formation of the bulb thus occurs due to interactions between the dermal papilla and bulge stem cells, causing the normally slow-cycling bulge stem cells to rapidly divide and differentiate during late telogen or anagen. After bulb formation, stem cells (now termed matrix cells) differentiate upward forming the inner root sheath (IRS) and hair shaft (Cotsarelis *et al.*, 1990). The IRS encloses the hair shaft in a rigid tube and guides it to the skin surface, while the outer root sheath (ORS) surrounds the IRS. After a period of growth the HF then enters the regression phase of catagen. Telogen sets in once full regression of the HF occurs, thus completing one cycle of the HF.

There are two possible explanations for hair growth cessation. One is that hair fiber production stops because the matrix cells have exhausted their proliferative capacities (Cotsarelis *et al.*, 1990; Lavker *et al.*, 1993). This view assumes that due to their involvement in continuous replication, matrix cells are transit-amplifying (TA) cells derived from the stem cells in the bulge. After each stem cell division, one of the two daughter cells leaves the bulge area and becomes a TA cell (a matrix cell; see Schofield *et al.*, 1983). As TA cells only possess transient proliferative properties, they eventually become exhausted and undergo terminal differentiation, or catagen (Straile *et al.*, 1961). The length of anagen is thus controlled by the matrix cells (Cotsarelis *et al.*, 1990). A second contrasting view is that stem cells in the bulge produce daughter matrix cells, and they continue to produce hair fibers until they are instructed to stop generating new daughter cells (Oshima *et al.*, 2001).

This view thus assumes that matrix cells possess identical characteristics to multipotent stem cells in the bulge. As stem cells possess unlimited proliferating abilities, they require instruction to stop generating new matrix cells.

Vitamin A (retinol) and its active derivatives (retinoids) are essential for a number of biological activities, including growth, vision, reproduction, hematopoiesis, immune system modulation, epithelial tissue homeostasis, and cell proliferation, differentiation, and apoptosis (Wolbach and Howe, 1925; reviewed in Mark *et al.*, 2009). These observations have led to the use of retinoids in the therapy of various skin diseases (Jones, 2005). Orally delivered retinoids are efficiently absorbed by the intestine [reviewed in (Napoli, 1999; Harrison, 2005)]. Dietary retinyl esters are hydrolyzed by retinol ester hydrolases (primarily pancreatic triglyceride lipase) in the intestinal lumen, yielding unesterified (free) retinol and the corresponding fatty acid (palmitic or stearic). Retinyl ester hydrolysis also requires the presence of bile salts that serve to solubilize the retinyl esters in mixed micelles and to activate the hydrolyzing enzymes. Retinol is taken up by the enterocyte and is re-esterified through the actions of the enzyme lecithin retinal acyltransferase (LRAT), which catalyzes the transacylation of a fatty acyl group from lecithin to retinol. In the intestine, cellular retinol-binding protein II (CRBP2), a member of the family of intracellular lipid-binding proteins, binds retinol with high affinity. This complex serves as a substrate for LRAT, thus facilitating retinol uptake into the body (reviewed in Harrison, 2005). The newly re-esterified retinyl ester is incorporated along with other dietary lipids into nascent chylomicrons that are then secreted from the enterocyte into the lymphatic system. Nascent chylomicrons enter general circulation through the thoracic duct, where lipoprotein lipase (LPL) converts chylomicrons to chylomicron remnants (Moise *et al.*, 2007). About 66-75% of retinyl esters contained in chylomicrons are taken up by the liver. These retinyl esters are

either secreted into the blood as (free) retinol bound to serum retinol-binding protein (RBP), or stored as retinyl ester in hepatic stellate cells. The remaining 22-33% of the retinyl esters from chylomicrons are taken up by target tissues including the skin, muscle, heart, lungs, reproductive organs, and bone marrow.

CRBPs function as specific carrier proteins for vitamin A metabolites (Ong *et al.*, 1994). These proteins bind all-*trans*-retinol and are found within cells important in vitamin A metabolism or function. Along with specific cellular locations of these proteins, slight differences in their binding to retinol suggest their different functions. In addition to being expressed in stellate cells of the liver, CRBP1 is expressed in many other organs and cell types including the kidney, eye, skin epithelia, and HF (Ong *et al.*, 1994; Everts *et al.*, 2007). This protein delivers retinol and retinal to specific binding sites within the nucleus and guides retinol and retinal to retinoid metabolizing enzymes, thus participating in the movement of retinol from the blood into target tissues (Ong *et al.*, 1988; reviewed in Napoli, 1999). In contrast, CRBP2 is expressed primarily in the intestine and is therefore involved in the intestinal absorption of vitamin A, directing retinol to LRAT as discussed previously. CRBP3 is expressed in the heart, muscle, adipose, and mammary tissues (Moise *et al.*, 2002). In some cases, the loss of one form of CRBP can be partly compensated for by the upregulation of other CRBPs.

Circulating free retinol (bound to RBP) is taken up by target tissues and oxidized to retinal by retinol dehydrogenases (ROLDHs) (reviewed in Napoli, 1999). Short-chain dehydrogenases/reductases (SDR family), such as dehydrogenase reductase 9 (Dhrs9), can additionally oxidize retinol bound to CRBP. Dhrs9 (identified as eROLDH in Rexer and Ong, 2002, also known as hRoDH-E2 in Markova *et al.*, 2003) is present in the majority of epithelial and epidermal cells, two sites where RA signaling is critical (Everts *et al.*, 2005;

Markova *et al.*, 2003). Due to the ability of SDRs to oxidize CRBP-bound retinol, these ROLDHs are thought to be the major physiological enzymes that catalyze the conversion of retinol to retinal, allowing for subsequent RA synthesis (Rexer and Ong, 2002; Markova *et al.*, 2003).

Retinal is converted to RA by retinal dehydrogenases (RALDH1, 2 and 3; gene names *Aldh1a1*, *Aldh1a2*, *Aldh1a3*) (Napoli, 1999). Cellular retinoic acid-binding proteins (CRABPs) are additional intracellular lipid binding proteins that bind only retinoic acid, despite structural similarities to CRBP (reviewed in Moise *et al.*, 2002). The generally believed function of CRABPs is to solubilize and protect RA in the cytosol. However, mounting evidence suggests that they also play a role in regulating signaling by RA (Budhu and Noy, 2002). CRABP2 was shown to transport RA from the cytosol to the nucleus, where it transfers RA to retinoic acid receptors (RARs) and enhances transcription. Evidence additionally infers that CRABP1 is involved in the breakdown of RA by directing RA to the CYP26 family of enzymes, but this has not been directly proven (reviewed in Moise *et al.*, 2002).

RA, specifically all-trans RA (atRA), was shown to be the most physiologically relevant form of vitamin A in keratinocytes (Kurlandsky *et al.*, 1994). The discovery of two classes of RA-binding transcriptional regulators, the retinoic acid receptors (RARs), and the retinoid X receptors (RXRs), explains most of the previously mentioned diverse biological activities of RA [reviewed in (Chambon, 1996; Leid *et al.*, 1992)]. Three subtypes of RARs are known: RARalpha (RARa), RARbeta (RARb), and RARgamma (RARg) (Gigeure *et al.*, 1987; Petkovich *et al.* 1987; Chambon, 1996). The functions of RARs were determined by Chambon and colleagues through knockout of the three RAR subtypes in mice, demonstrating that RAR is instrumental to RA signaling during embryonic development

(Lohnes *et al.*, 1995). All RAR single-null mutant males were viable but displayed some aspects of the postnatal and fetal vitamin A deficiency syndromes. Unlike RAR single mutants, double-null mutants died either *in utero* or shortly after birth [reviewed in (Germain *et al.*, 2006; Mark *et al.*, 2007)]. Three subtypes of RXR (RXRalpha (a), beta(b), and gamma(g)) also exist (reviewed in Mark *et al.*, 2009). As RARs are type II nuclear receptors, they function along with the retinoid X receptor (RXR) by forming a heterodimeric complex. RAR-RXR heterodimers selectively control target gene expression and differentiation controlled by retinoids. More specifically, RARg-RXRa heterodimers are necessary for growth arrest and endodermal differentiation.

As RA synthesis is associated with the aforementioned enzymes and binding proteins, their expression throughout the hair cycle indicates sites of RA, while the presence of receptors indicates putative sites of action. One study showed the localization of RA biosynthesis and signaling components throughout the HF during the murine hair cycle (Everts *et al.*, 2007). Overall, all of the RAR subtypes were widely present and were localized in the epidermis and the HF. RARa and RARg were ubiquitously expressed in HF throughout the hair cycle, while RARb showed a unique localization pattern. The whole system necessary for RA synthesis and signaling was present during early to mid-anagen (II - IV). Specifically, RA synthesis enzymes (Dhrs9, ALDH1a1-3) along with RARs and CRABP2 increased. ALDH1a2 and RARs decreased during late anagen/early catagen (VI/I), but returned by late catagen (IV). CRBPI increased during anagen VI/catagen I and remained intense throughout catagen VI. RARs and Dhrs9 decreased by catagen VII in the cycling portion of the HF. During telogen, RARs were prominent in the cycling portion of the HF. As RA signaling and synthetic enzymes were distributed throughout and

surrounding the HF during the hair cycle, this suggests multiple roles for RA during this process.

*Wnt* genes function in short-range signaling and therefore play a role during development of many tissues. They regulate cell fate, differentiation, proliferation, and mobility in skeletal muscle, kidney, the nervous system, and the HF (reviewed in Peifer and Polakis, 2000). WNT signaling proteins are secreted factors that contain a dozen or so members and are grouped into two classes based on function (Reddy *et al*, 2001). Class I WNTs exert the aforementioned effects through a canonical signaling pathway. When WNT is present (**Figure 3**), the canonical pathway begins by binding of WNTs to their frizzled (Fz) and low-density lipoprotein receptor-related protein receptor complexes on the cell surface. This binding inhibits glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ), thus increasing levels of free  $\beta$ -catenin in the cell. Disheveled protein (DVL) stabilizes cytosolic  $\beta$ -catenin, allowing it to translocate to the nucleus.  $\beta$ -catenin is a structural component of adherens junctions in the cell membrane, linking E-cadherins to the cytoskeleton by binding to  $\alpha$ -catenin. Once in the nucleus,  $\beta$ -catenin forms transcriptional complexes with the N-terminus of members of the lymphoid enhancer factor/T cell factor (LEF/TCF) family of transcription factors. This binding controls the transcription of WNT target genes (Wodarz and Nusse, 1998). Therefore, nuclear  $\beta$ -catenin marks sites of active WNT signaling in HF. Class II WNTs function via less well-known pathways and mediate proliferation, cell movements in gastrulation, and cell polarity (Millar *et al*, 1999). Regulation of cell movements and polarity requires DVL but not  $\beta$ -catenin. In the absence of WNT (**Figure 4**), the cytoplasmic pool of  $\beta$ -catenin that is not complexed with cadherins is rapidly phosphorylated at the N-terminus by GSK-3 $\beta$ . This complex is marked for proteasome degradation, resulting in its breakdown.

The WNT/  $\beta$ -catenin signaling pathway has been proposed to function in HF

Figure 3: Canonical signaling pathway in the presence of WNT

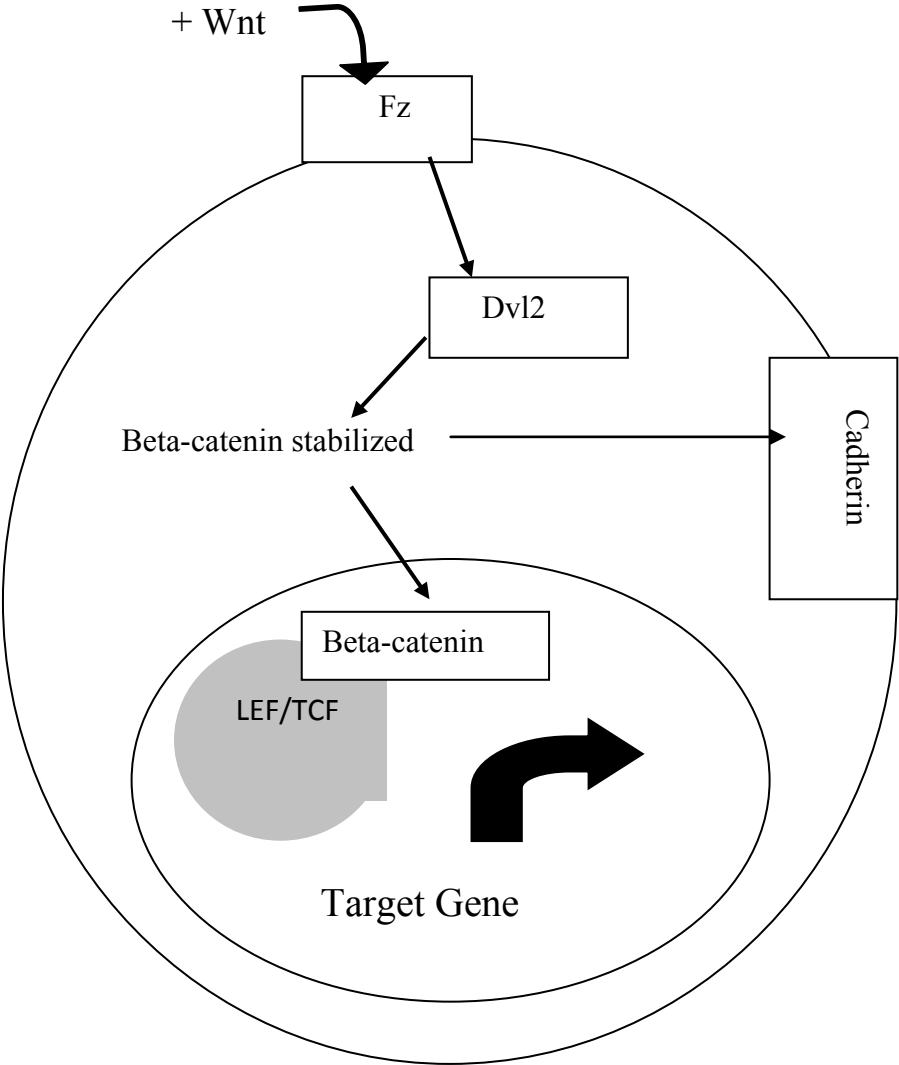
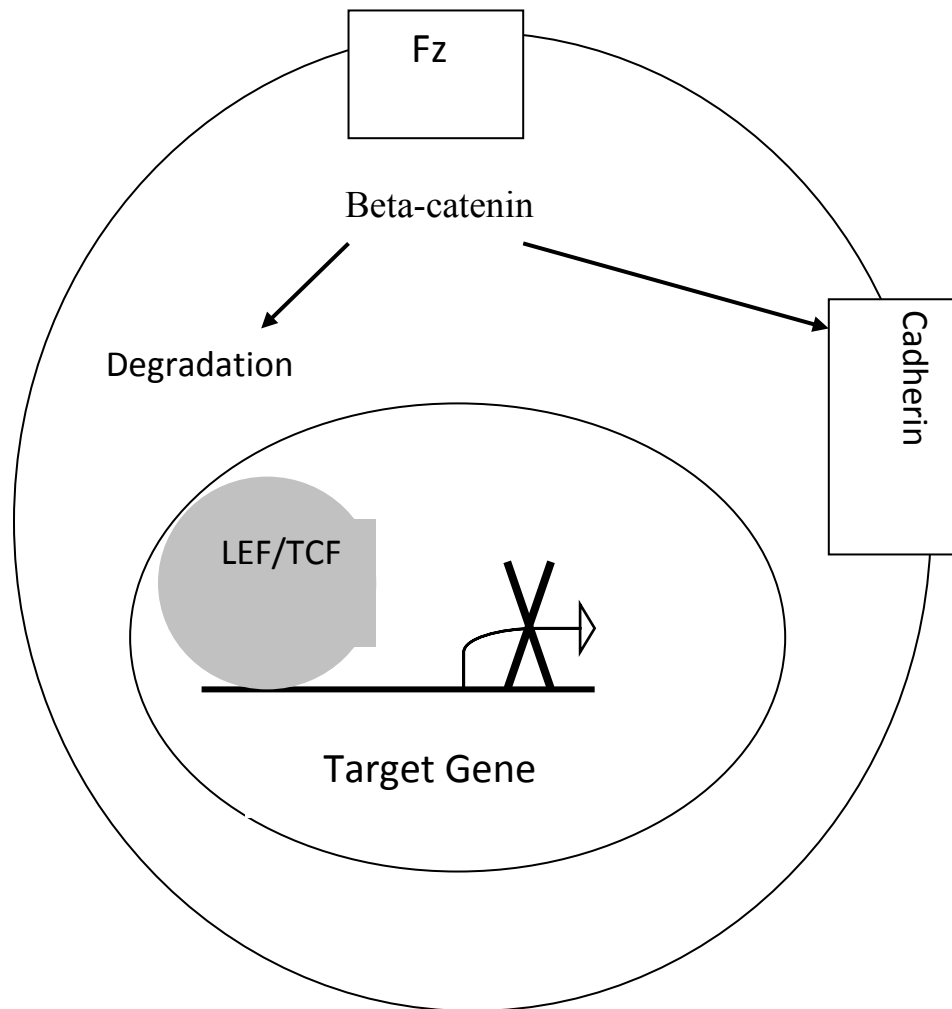




Figure 4: Signaling pathway in the absence of WNT

No WNT



morphogenesis and differentiation (Kishimoto *et al.*, 2000). When exposed to WNTs, dermal papilla cells in culture retained their ability to induce follicles when transplanted back into mice. Substantial evidence suggests that  $\beta$ -catenin signaling is important in epidermal development, homeostasis, and disease (reviewed by Fuchs *et al.*, 2001). Mice with HF-specific deletion of  $\beta$ -catenin (Huelsen *et al.*, 2001) or overexpression of an inhibitor of WNT signaling (Andl *et al.*, 2002) did not develop HF. Similarly, when the WNT transcription factor gene, *Lef-1*, was ablated in the epidermis, mice did not form hair follicles (van Genderen *et al.*, 1994). On the contrary, mice overexpressing WNT signaling via a stabilized  $\beta$ -catenin under control of keratin 14 (*K14*) promoter experienced de novo HF morphogenesis, produced an excess of HF, and developed tumors (Gat *et al.*, 1999). Additionally, WNT/ $\beta$ -catenin signaling plays a role in anagen initiation during the hair cycle, as transient activation of the  $\beta$ -catenin pathway in the epithelium is sufficient to initiate the anagen phase to induce new HF (Lo Celso *et al.*, 2004; Shimizu and Morgan, 2004). Furthermore, the first postnatal anagen does not occur in mice in which  $\beta$ -catenin expression is not present in the skin (Huelsen *et al.*, 2001). In addition, an increase in WNT signaling in the bulge of the HF at onset of anagen in the WNT reporter mice (transgenic TOPGAL) was observed (DasGupta and Fuchs, 1999). Moreover, several WNTs, including 10a and 10b, are expressed in the epidermis of postnatal hair follicles at anagen onset, but not in resting follicles (Reddy *et al.*, 2001).

RA has been shown to regulate WNT-signaling pathways in various tissues and cell lines (DasGupta and Fuchs, 1999; Huelsen *et al.*, 2001; Reddy *et al.*, 2001; Lo Celso *et al.*, 2003; Shimizu and Morgan, 2004), but no studies have examined regulation of this pathway in skin or hair follicles. Additionally, the localization pattern of RA synthesis and signaling components during early anagen in proliferating and migrating keratinocytes was similar to

that of various WNT-signaling factors (Everts *et al* 2007, Reddy *et al.*, 2001). The specific role of RA in the hair cycle is currently under investigation, as RA has been found to have either antagonistic or synergistic effects on WNT signaling. Evidence for mutual antagonism between  $\beta$ -catenin and RA exists (Mongan and Gudas, 2007).  $\beta$ -catenin upregulates *CYP26A1*, degrading RA through oxidation and, conversely, RAR interacts with  $\beta$ -catenin and competitively inhibits TCF binding sites in vitro (Easwaran *et al*, 1999; Mongan and Gudas, 2007). RA was also found to inhibit WNT signaling by positively regulating the WNT inhibitors Disabled-2, Dkk1, and Sox1 (Shibamoto *et al*, 2004; Zhuang *et al*, 2003). Some of these WNT inhibitors are also upregulated in the hair follicle stem cell (Tumbar *et al*, 2004; Morris *et al*, 2004), suggesting that RA may be associated with stem cell regulation. RA has also been shown to have a synergistic effect with WNT signaling in mouse mammary epithelial cells (C57MG) that conditionally express WNT (Szeto *et al*, 2001; Tice *et al*, 2002) as well as totipotent teratocarcinoma cells (Liu *et al*, 2002). This association was accompanied by an increase in RAR $\gamma$  by *Wnt1* as well as a necessity of RA for *Wnt1* transcriptional activation. These studies suggest that there is a complex interaction between RA and WNT signaling. However, the specific effects of endogenous retinoids on hair follicles remain uncertain. Research has not been conducted to determine the localization of WNT signaling in all 17 stages of the hair cycle. Additionally, research focused on the interactions of RA and WNT falls short of explaining the specific role of RA in regulating the hair cycle. Taking all of this into account, my hypothesis is that retinoic acid (RA) inhibits WNT signaling. To gain a better understanding of how RA interacts with WNT signaling, we performed three preliminary studies: 1) Determined where and when during the hair cycle nuclear  $\beta$ -catenin co-localizes with RA synthesis; 2) Determined a drug and dose

that inhibits RA synthesis in the skin in order to determine if RA inhibits WNT signaling; 3) Determined the easiest and most cost effective manner to measure WNT signaling.

## **Materials and Methods**

### **Tissue acquisition for localization of WNT signaling:**

Paraffin embedded tissue sections from 24 female C3H/HEJ mice were obtained from John P. Sundberg at The Jackson Laboratory (Bar Harbor, ME). These mice were wax stripped in order to induce anagen and synchronize the hair cycle, as described previously (Everts *et al.*, 2007). Skin samples were harvested from two C3H/HeJ wax-stripped mice at each of the following time points: 0, 3, 6, 9, 12, 15, 18, 21, and 24 days after depilation. These tissues were sectioned at 5-6  $\mu\text{m}$ , placed on microscope slides, and stained with hematoxylin and eosin for routine histopathologic analyses.

### **Mice treatments for drug study:**

Thirty-two C57BL/6J adult mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in a temperature and light-controlled room. They were fed a chow diet (Harlen Tekland 7912, with vitamin A acetate at 29.63 IU/gram), unless noted below, provided water ad libitum, and were allowed to acclimate for at least one week before use in these experiments. Studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and all mouse studies were approved by The Ohio State University IACUC. At 9 weeks old, the mice were anesthetized with isoflurane (Abbott Laboratories, N Chicago, IL) and pre-treated with the analgesic carprofen (Rimadyl, Pfizer Inc., New York, NY) diluted to 0.5 mg/ml and injected subcutaneously at 0.1 ml/g body weight. All dorsal skin follicles were painted with warm wax (Surgi-wax; Ardell International, Los Angeles, CA) that was peeled off after hardening, thus depilating all dorsal

skin hair fibers and synchronizing the hair cycle. After depilation, the mice were placed on a heating pad. This warmed them until they completely recovered from anesthesia, at which point they were returned to their housing location. After six days, a time span determined by the localization of nuclear  $\beta$ -catenin pilot study, the mice were anesthetized with isoflurane. Acetone (vehicle control) or one of 4 doses of either citral (0.01875, 0.0375, 0.075, 0.15 nmol/g body weight; Sigma, St. Louis, MO) or disulfiram (0, 0.56, 1.12, 2.24, 4.48  $\mu$ mol/g body weight; Sigma) was applied to the dorsal side of each mouse under the hood. Citral is a monoterpene aldehyde and competitive substrate for retinal dehydrogenase, while disulfiram is a sulfhydryl-group cross-linking agent, and both of these drugs are potent inhibitors of retinoic acid synthesis. After topical application, the mice were placed on a heating pad and monitored until fully recovered from anesthesia, at which time they were returned to their housing location. Four hours later mice were euthanized by CO<sub>2</sub> inhalation. Dorsal skin was fixed overnight in zinc alcohol buffered paraformaldehyde, transferred to 70% ethanol, processed routinely, embedded in paraffin, sectioned at 5-6  $\mu$ m, placed on microscope slides for immunohistochemistry (IHC) and routine histology, collected in RNAlater (Ambion, Austin, TX) for QPCR, or frozen in OTC.

### **Transgenic mice:**

Two additional adult mice, one Tg (Fos-lacZ) 34EFu/J mouse and one wild type (Wt) CD-1 control were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed as previously described. The Tg (Fos-lacZ) 34EFu/J and Wt CD-1 mice were anesthetized with isoflurane and wax-stripped as previously described, thus depilating all dorsal skin hair fibers in order to induce and synchronize the hair cycle. Six days after depilation (time period was determined by the localization of nuclear  $\beta$ -catenin pilot study), the Tg (Fos-lacZ) 34EFu/J

and Wt CD-1 mice were euthanized by CO<sub>2</sub> inhalation. Dorsal skin was fixed for immunohistochemistry (IHC) and routine histology as previously described, collected in RNAlater (Ambion, Austin, TX) for QPCR, or frozen in OTC.

### **IHC:**

Sections were pretreated with 3% hydrogen peroxide, blocked with 3% Bovine Serum Albumin (BSA) plus 1.28% normal goat serum, and incubated with primary antibody (Rabbit anti-beta-catenin, Cat #ABI9022 from Chemicon International, Billerica, MA; Rabbit anti-RAR- $\beta$ , from Santa Cruz Biotechnology Inc., Santa Cruz, CA; Rabbit anti-beta-galactosidase, Abcam Inc., Cambridge, MA) overnight at 4°C. Tissues were incubated with a biotinylated anti-rabbit secondary antibody, an anti-biotin IgG-conjugated with horseradish peroxidase tertiary antibody, and then were stained with 3-amino-9-ethylcarbazole (AEC) + substrate-chromagen (outlined in Everts *et al.*, 2007). Hydrogen peroxide and BSA were obtained from Fisher Scientific (Waltham, MA), normal goat serum and secondary antibody were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA), tertiary antibody was obtained from Bethyl labs (Montgomery, TX), and AEC+ was obtained from Dako (Carpinteria, CA).

### **Quantitative (Real-Time) Polymerase Chain Reaction (QPCR):**

QPCR was conducted on the dorsal skin placed in RNAlater once collected from the euthanized mice. *Crbp* (*Rbp1*) and *Rarb* were used as indicators for retinoic acid (RA) activity as these are classical target genes (Napoli *et al.* 1991; Ruberte *et al.* 1993). An RNeasy Fibrous Tissue Mini Kit (Qiagen Inc., Valencia, CA) was used to isolate purified RNA. In place of a tissue rupter/lyser, a mini-bead beater (BioSpec Products, Bartlesville,

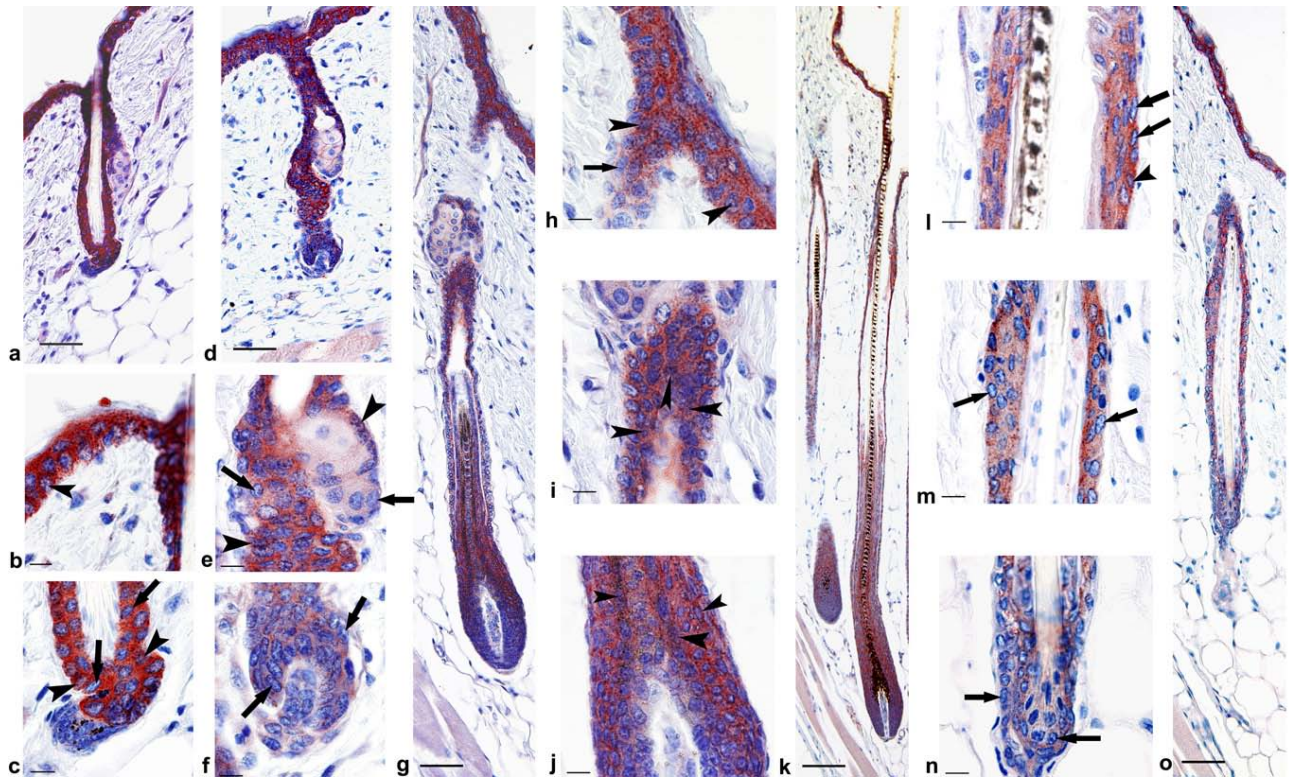
OK) was used to break up tissue, and volumes were adjusted to accommodate this. RNA levels were measured on a UV/Vis spectrophotometer (nanodrop ND-1000, Thermo Scientific, Wilmington, DE). Using a High-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) and Mastercycler ep (Authorized Thermocycler from Eppendorf), 2 µg of this total RNA were converted to single-stranded cDNA that was suitable for qPCR applications. qPCR reactions were then performed using 50 ng cDNA in the TaqMan Gene Expression Assay kit for *Rarb* and *Rbp1* (Applied Biosystems, Foster City, CA) and the Applied Biosystems 7300 qPCR machine. Relative expression was calculated using the ddCT method (outlined in Bookout *et al.*, 2006) using Beta-actin as the endogenous control (Mouse ACTB, Applied Biosystems).

## **Results:**

### **Nuclear β-catenin peaked 6 days after wax stripping, when the hair follicles were in anagen IIIc or IV.**

To determine a specific point in the hair cycle where WNT signaling co-localized with RA synthesis and signaling, we performed IHC with an antibody against β-catenin on previously collected mice tissue that had been wax stripped and collected every 3 days for 24 days. Hair follicles were then classified by stage (according to Muller-Rover *et al.*, 2001). The presence of nuclear localization of β-catenin marks sites of active WNT signaling in hair follicles (**Figure 5**, black arrowhead). This was compared to the previous localization of RA synthesis and signaling (Everts *et al.*, 2007) to determine when WNT signaling co-localized with RA synthesis during the hair cycle.

During telogen (**Figure 5 a-c**), which was 0 or 24 days after wax-stripping, nuclear β-catenin appeared in the epidermis and a few outer root sheath cells near the bulge and dermal



**Figure 5: IHC determination of nuclear localization and exclusion of  $\beta$ -catenin in hair follicles throughout the hair cycle.** Nuclear localization and exclusion of  $\beta$ -catenin in hair follicles throughout the hair cycle from C3H/HEJ mice that were wax-stripped to induce anagen and collected every 3 days for 24 days. Telogen hair follicles (**a-c**), early anagen (II) hair follicles (**d-f**), mid-anagen (IIIc) hair follicles (**g-j**), late anagen (VI) hair follicles (**k-l**), and catagen V hair follicles (**m-o**). Black arrowhead, nuclear localization; black arrow, nuclear exclusion. For **a, d, g, k, and o**, bar = 50.3  $\mu$ m, for **b-c, e-f, h-j, l-n**, bar = 10.1  $\mu$ m.



papilla.  $\beta$ -catenin was excluded from the nucleus in the dermal papilla and most outer root sheath, bulge cells, and cells in the sebaceous gland. In early anagen I-IIIB (**Figure 5 d-f**), which was 3 days after wax-stripping, nuclear  $\beta$ -catenin remained in the epidermis and outer root sheath, and appeared on the edge of the sebaceous gland and in a few bulge cells.  $\beta$ -catenin was excluded from the nucleus in the dermal papilla, matrix cells, proliferated keratinocytes, and most bulge cells. During mid-anagen IIIc-IV (**Figure 5 g-j**), which was 6 days after wax-stripping, nuclear  $\beta$ -catenin peaked and was strong in the outer root sheath, inner root sheath, bulge cells, and proliferating keratinocytes.  $\beta$ -catenin was also present in the nucleus in some dermal papilla and matrix cells of the bulb. It was difficult to determine whether  $\beta$ -catenin was excluded in most bulb cells and the sebaceous gland, as there may have been some background staining in these areas. By late anagen V-VI (**Figure 5 k-l**), which was 9-15 days after wax-stripping,  $\beta$ -catenin was nuclear excluded everywhere except the bulb and some outer root sheath and inner root sheath cells near the sebaceous gland. In early catagen (I-III), which was 18 days after wax stripping,  $\beta$ -catenin was nuclear excluded everywhere except the bulb and outer root sheath cells near the bulge. By catagen IV-V (**Figure 5 m-o**), which was 18 days after wax stripping,  $\beta$ -catenin was nuclear excluded everywhere in the hair follicles. Lastly, during late catagen (VI-VIII), which was 21 days after wax stripping,  $\beta$ -catenin remained nuclear excluded everywhere except for a few outer root sheath cells.

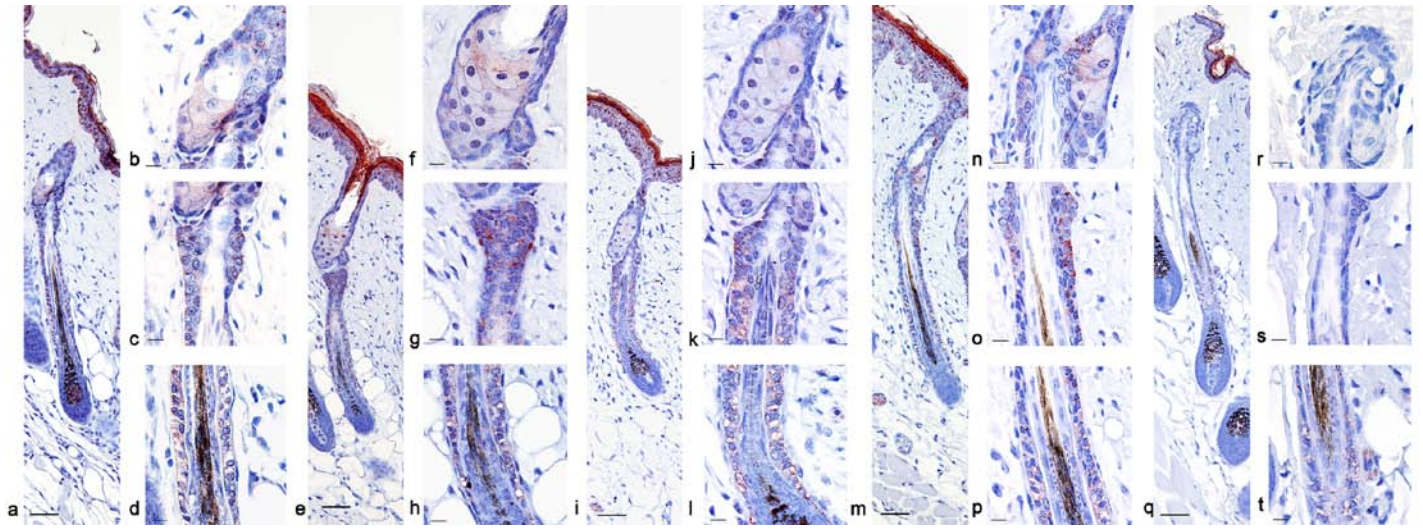
#### **Citral was the best inhibitor and resulted in WNT signaling in sebocytes.**

To determine a drug and dose to use that topically inhibited retinoic acid (RA) synthesis, citral, disulfiram, or acetone (control) was applied to the dorsal side of mice 6 days after wax stripping, when WNT signaling peaked in the nuclear localization of  $\beta$ -catenin study. QPCR

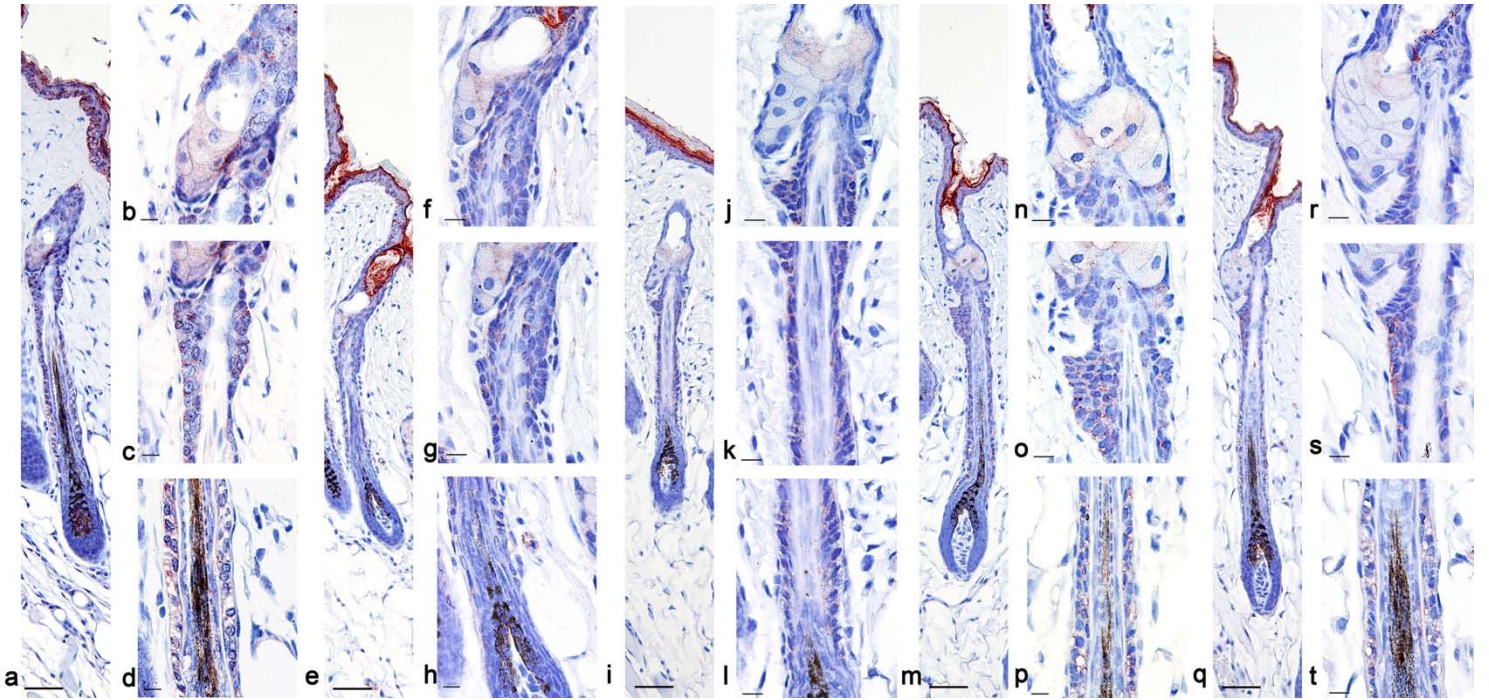
analysis using either *Crpp(Rbp1)* or *Rarb* RA target genes as markers for RA activity in full thickness dorsal mouse skin did not provide significant differences between any of the doses of citral or disulfiram (data not shown).

The presence of RARb marks several sites of RA activity in hair follicles. IHC conducted on anagen IIIc/IV hair follicles with an antibody against RARb (**Figure 6 a-d**) determined that RARb localized to the dermal papilla, outer root sheath, inner root sheath (Henle's layer), bulge cells, sebaceous gland, and proliferated keratinocytes in the acetone control. The highest dose of citral (0.15 nmol/g body weight; **Figure 6 q-t**) showed a strong reduction of RARb in the sebaceous gland, bulge cells, and proliferated keratinocytes. However, this dose did not inhibit RARb protein expression in the dermal papilla, inner root sheath, or outer root sheath. Remaining doses of citral (0.075, 0.0375, and 0.01875 nmol/g body weight; **Figure 6 e-p**) did not provide significant differences in RARb expression and were similar to the acetone control. No dose of citral completely inhibited RARb expression. The highest dose of disulfiram (4.48  $\mu$ mol/g body weight, **Figure 7 q-t**) showed reduced RARb protein expression in the sebaceous gland compared to the control (**Figure 7 a-d**). However, this dose did not inhibit RARb in the dermal papilla, outer root sheath, inner root sheath (Henle's layer), proliferated keratinocytes, or bulge cells. Remaining doses of disulfiram (2.24, 1.12, and 0.56  $\mu$ mol/g body weight) (**Figure 7 e-p**) did not provide significant differences in RARb protein expression and were similar to the acetone control. No dose of disulfiram completely inhibited RARb, and the highest dose was not as effective of an inhibitor as the highest dose of citral.

IHC conducted on anagen IIIc/IV hair follicles, and with an antibody against  $\beta$ -catenin (**Figure 8 a-d**), showed nuclear exclusion of  $\beta$ -catenin in the outer root sheath, bulge cells, and sebaceous gland in the acetone control. IHC conducted on anagen IIIc/IV hair follicles,



**Figure 6: IHC determination of RARb inhibition via citral compared to the acetone control in anagen IIIc/IV hair follicles.** RARb inhibition in anagen IIIc/IV hair follicles from C57BL/6J mice that had acetone (vehicle control) or 1 of 4 doses of citral topically applied to their dorsal skin 6 days after wax-stripping, when WNT signaling peaked in the previous study. Acetone (**a-d**), citral 0.01875 nmol/g body weight (**e-h**), citral 0.0375 nmol/g body weight (**i-l**), citral 0.075 nmol/g body weight (**m-p**), and citral 0.15 nmol/g body weight (**q-t**) in anagen IIIc/IV hair follicles. Red color, RARb expression. For **a**, **e**, **i**, **m**, and **q**, bar = 50.3  $\mu$ M, for **b-d**, **f-h**, **j-l**, **n-p**, and **r-t**, bar = 10.1  $\mu$ M.



**Figure 7: IHC determination of RARb inhibition via disulfiram compared to the acetone control in anagen IIIc/IV hair follicles.** RARb inhibition in anagen IIIc/IV hair follicles from C57BL/6J mice that had acetone (vehicle control) or 1 of 4 doses of disulfiram topically applied to their dorsal skin 6 days after wax-stripping, when WNT signaling peaked in the previous study. Acetone (**a-d**), disulfiram 0.56  $\mu\text{mol/g}$  body weight (**e-h**), disulfiram 1.12  $\mu\text{mol/g}$  body weight (**i-l**), disulfiram 2.24  $\mu\text{mol/g}$  body weight (**m-p**), and disulfiram 4.48  $\mu\text{mol/g}$  body weight (**q-t**) in anagen IIIc/IV hair follicles. Red color, RA expression. For **a**, **e**, **i**, **m**, and **q**, bar = 50.3  $\mu\text{m}$ , for **b-d**, **f-h**, **j-l**, **n-p**, and **r-t**, bar = 10.

and with an antibody against  $\beta$ -catenin (**Figure 8 e-h**), showed nuclear  $\beta$ -catenin in the bulge cells and sebaceous gland of the mice that received the highest doses of citral and disulfiram.

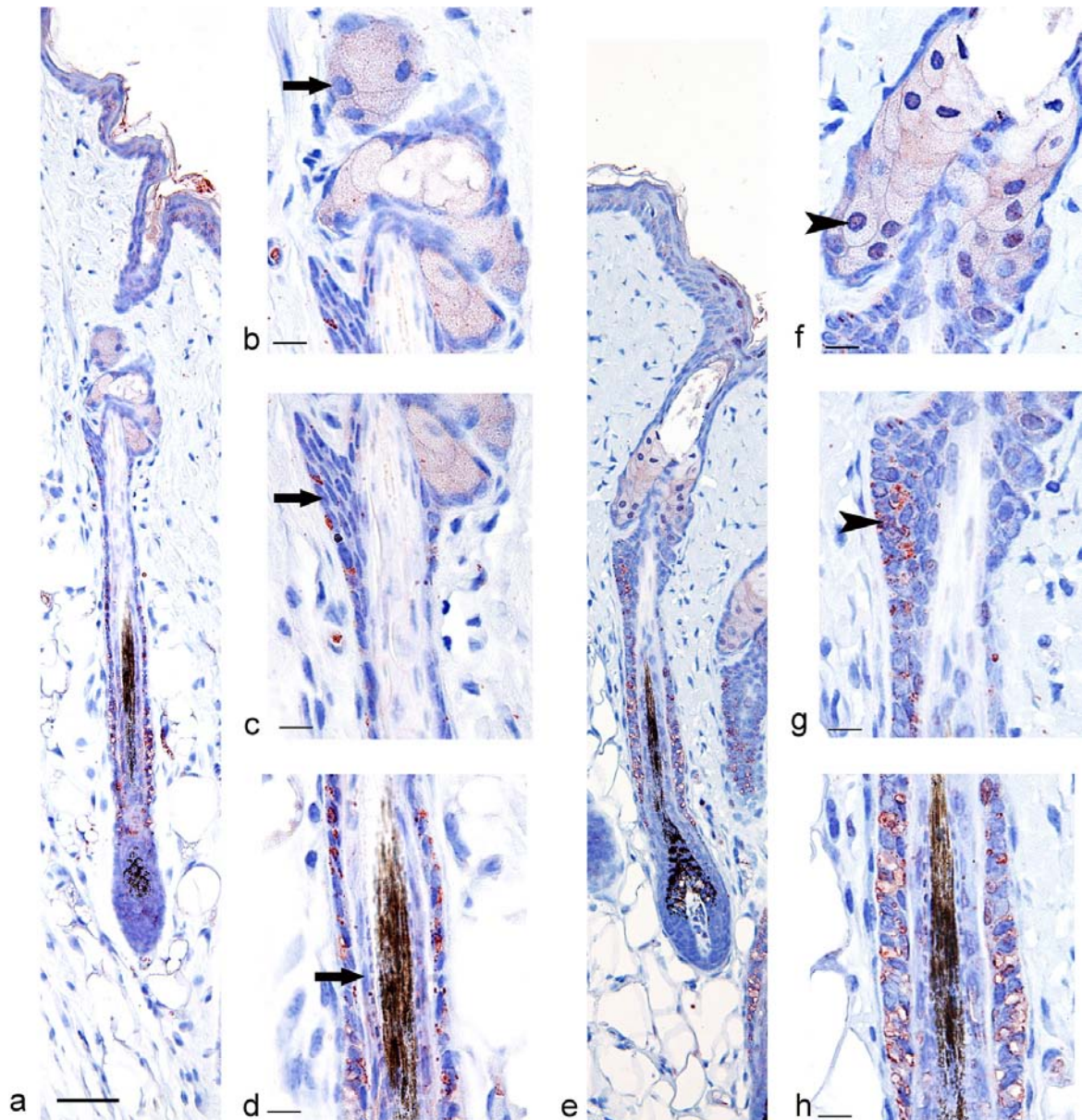
### **Nuclear $\beta$ -catenin and WNT reporter mice provided identical results.**

To easily visualize WNT signaling in the most cost effective manner, two additional adult mice were wax stripped, and 6 days later, they were euthanized and dorsal skin collected.  $\beta$ -galactosidase in the WNT reporter mouse (TOPGAL) and nuclear  $\beta$ -catenin in any mouse marked sites of active WNT signaling. IHC conducted on anagen IV hair follicles with an antibody against  $\beta$ -galactosidase (**Figure 9 a-d**) found strong expression of  $\beta$ -galactosidase in the bulb and inner root sheath, and faint  $\beta$ -galactosidase expression in the bulge cells of the TOPGAL mice. IHC conducted on anagen IV hair follicles with an antibody against  $\beta$ -catenin (**Figure 9 e-f**) also showed nuclear  $\beta$ -catenin in the bulb, inner root sheath, and bulge cells. However, it was more difficult and time consuming to determine nuclear localization of  $\beta$ -catenin in normal mice versus the presence of  $\beta$ -galactosidase in the transgenic reporter mice.

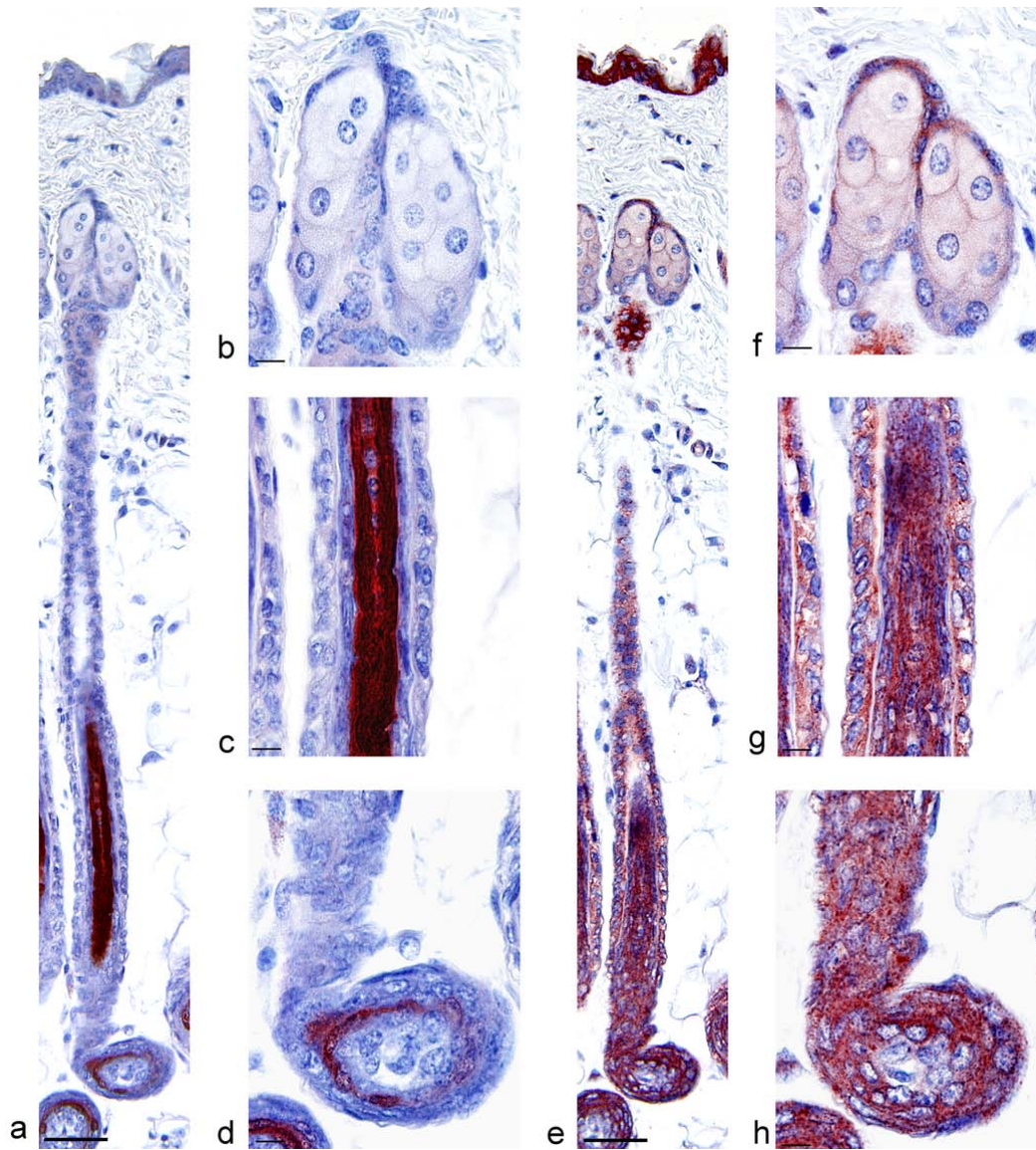
### **Discussion:**

To gain a better understanding of how RA interacts with WNT signaling, we performed three preliminary studies. We found that nuclear  $\beta$ -catenin peaked 6 days after wax stripping, when the hair follicles were in anagen IIIc or IV. We also determined that citral provided the strongest inhibition of RAR $\beta$ , which increased WNT signaling in sebocytes and some bulge cells. Furthermore, nuclear  $\beta$ -catenin and WNT reporter mice produced identical localization patterns. As aberrant WNT/ $\beta$ -catenin signaling has been





**Figure 8: IHC determination of nuclear localization and exclusion of  $\beta$ -catenin in anagen IIIc/IV hair follicles when RA is inhibited.** Nuclear localization and exclusion of  $\beta$ -catenin in anagen IIIc/IV hair follicles from C57BL/6J mice that were wax-stripped to induce anagen and had acetone control (**Figure 8 a-d**), the highest dose of citral (0.15 nmol/g body weight; **Figure 8 e-h**), or the highest dose of disulfiram (4.48  $\mu$ mol/g body weight; not pictured) topically applied to their dorsal skin. Black arrowhead, nuclear localization; black arrow, nuclear exclusion. For **a** and **e**, bar = 50.3  $\mu$ m, for **b-d** and **f-h**, bar = 10.1  $\mu$ m.



**Figure 9: IHC determination of  $\beta$ -galactosidase expression versus nuclear  $\beta$ -catenin localization in anagen IIIc/IV hair follicles.** IHC determination of  $\beta$ -galactosidase expression versus nuclear  $\beta$ -catenin localization in anagen IIIc/IV hair follicles of a transgenic (TOPGAL) mouse that was wax-stripped to induce anagen and dorsal skin collected 6 days later.  $\beta$ -galactosidase (**Figure 9 a-d**): red stain;  $\beta$ -catenin (**Figure 9 e-g**): black arrowhead, nuclear  $\beta$ -catenin localization; black arrow, nuclear  $\beta$ -catenin exclusion. For **a** and **e**, bar = 50.3  $\mu$ m, for **b-d** and **f-h**, bar = 10.1  $\mu$ m.

implicated in many types of cancers, these findings play a role in determining the importance of how RA interacts with WNT signaling.

As  $\beta$ -catenin marks sites of active WNT signaling, we concluded that WNT signaling peaks 6 days after wax stripping, when normal hair follicles are in mid-anagen (IIIc/IV). WNT signaling is strongest during mid-anagen in the differentiating inner root sheath, outer root sheath, bulge cells, and proliferating keratinocytes. It is also present in some dermal papilla and matrix cells of the bulb. The majority of these localization sites are consistent with previous studies, which found that specific *Wnt* genes (Reddy *et al.*, 2001; Millar *et al.*, 1999), nuclear  $\beta$ -catenin (Tsuji *et al.*, 2001; Ridanpaa *et al.*, 2001; Moreno-Bueno *et al.*, 2001), or frizzled WNT receptors (Reddy *et al.*, 2004) were present during mature anagen hair follicles. However, no prior research has been conducted to determine the localization of WNT signaling in all 17 stages of the hair cycle. We therefore wanted to perform an experiment that would pinpoint the exact stage during which WNT signaling was the strongest. RA synthesis and signaling components peaked in mid-anagen, 6 days after wax stripping (Everts *et al.*, 2007), demonstrating similar localization patterns to what we found for WNT signaling. These conclusions led us to collect the remainder of the dorsal mouse skin 6 days after wax stripping, as the overall objective of our research was to determine how RA altered WNT signaling.

IHC analysis determined that the highest dose of citral best inhibited RAR $\beta$  in the sebaceous gland, bulge cells, and proliferating keratinocytes. As RAR $\beta$  marks sites of RA signaling, we concluded that RA signaling was inhibited in these sites. However, the highest dose of citral did not provide complete inhibition of RA signaling, particularly in the dermal papilla, inner root sheath, and outer root sheath. It is possible that outer root sheath localization may be background staining, as previous studies did not find RAR $\beta$  in this area



of the hair follicle (Everts *et al.*, 2004, 2007). Another possible explanation for the lack of complete RA inhibition is that the doses for citral were not what we originally intended to use. The highest dose of citral was 1000-fold lower than the lethal dose (LD) 50, or the dose where 50% of the mice do not survive. These findings suggest that higher doses are physiologically safe to use and will most likely result in stronger RA inhibition. Further studies should be conducted in order to determine the optimal amount to accomplish complete RA inhibition.

Previous studies in our lab determined that RARb has a similar co-localization pattern to retinal dehydrogenase1 and 2 (RALDH1 and RALDH2/ALDH1A1 and ALDH1A2), but not ALDH1A3 (Everts *et al.*, 2004, 2007). During anagen and early catagen, ALDH1A1 localized to the sebaceous gland, ALDH1A2 localized to the bulge region, and ALDH1A3 localized to either the mesenchymal dermal papilla or epithelial pre-inner root sheath cells in the bulb region. Our finding that RARb was present in the sebaceous gland and bulge cells of the acetone control during mid-anagen is consistent with these previous studies. This allows us to assume that citral was actually inhibiting RARb in these sites, as previously mentioned. However, usage of the RARb antibody is limiting, as it does not localize to all sites where RA synthesis and signaling components have been detected. RARb does not co-localize with ALDH1A3, and therefore is not present in the dermal papilla or pre-inner root sheath cells in the bulb. Consequently, it is not possible to determine whether RA activity is inhibited in these sites. Retinoic acid response element (RARE) reporter mice detect all RA signaling in hair follicles. Therefore, the usage of these mice in future studies would allow for a better indication of sites where RA has been inhibited.

QPCR analysis of RNA from whole dorsal mouse skin did not provide significant differences between any of the doses of citral or disulfiram when using *Crpp(Rbp1)* or *Rarb*

as target genes for RA activity. This finding may be due to the fact that whole skin RNA was used instead of the specific hair follicles from the dorsal tissue. RARb does not detect all sites of RA signaling in the hair follicle, and the hair follicle is only a small fraction of the skin. Consequently, using whole skin RNA may have diluted the amount of detectable RARb, thereby making it difficult to detect significant differences in RA inhibition.

In the acetone control mice, IHC analysis showed nuclear exclusion of  $\beta$ -catenin. In mice that received the highest dose of citral, IHC analysis showed nuclear  $\beta$ -catenin in the sebaceous gland and some bulge cells. As previously mentioned, RA was inhibited in the bulge cells and sebaceous glands of these mice. Along with the fact that nuclear  $\beta$ -catenin marks sites of active WNT signaling, these results further indicate that RA inhibits WNT signaling. Our hypothesis therefore appears to be on the right track. However, these results are only representative of a small sample size. Once an optimal dose of citral is found to completely inhibit RA, a larger study should be performed with more mice in order to confirm that RA inhibits WNT signaling in a statistically significant sample size.

The  $\beta$ -galactosidase localization pattern in the WNT reporter mice was the same as we saw with the  $\beta$ -catenin antibody. However, it was more difficult and time consuming to determine nuclear localization of  $\beta$ -catenin versus the presence of  $\beta$ -galactosidase in the hair follicles. This conclusion will help us decide which mice are best to use for future studies. When monetary resources are low, the  $\beta$ -catenin method would be better to use, as the TOPGAL reporter mice are more expensive than normal mice. On the other hand, when adequate monetary resources are available, the  $\beta$ -galactosidase method would be better to use, as it takes less time to detect WNT signaling in these mice.

WNT/ $\beta$ -catenin signaling is important in determining stem cell fate during the hair cycle, and regulates whether sebaceous glands or hair follicles are formed (Merrill *et al.*,

2001; Fuchs and Horsley, 2008). When aberrant WNT/ $\beta$ -catenin signaling is inhibited in the sebaceous gland (but not other areas of the hair follicle), hair follicles form from those cells. However, aberrantly induced WNT/ $\beta$ -catenin signaling in the sebaceous gland leads to proliferation of sebocytes at the expense of hair follicle formation. As we observed WNT signaling in the sebaceous gland and some bulge cells of dorsal skin when RA was not present, this implies that RA normally regulates and inhibits WNT/ $\beta$ -catenin signaling in these areas of the hair follicle. This implication is supported by a previous study, which found that retinoic acid decreased the activity of the  $\beta$ -catenin-LEF/TCF signaling pathway, a key component of the WNT signaling pathway in MCF7 breast cancer cells, retinoid-sensitive adenomatous polyposis coli (APC)-mutant colon cancer (Caco-2) and retinoid-sensitive APC-mutant human colon adenocarcinoma grade II (HT29) cells (Easwaran *et al.*, 1999). One implication for aberrant WNT/ $\beta$ -catenin signaling is that many tumors and cancers in the skin and body, as well as acne, arise from aberrant sebocyte proliferation (Widelitz, 2004; Fodde and Brabletz, 2007; Slavik *et al.*, 2007; Gehrke *et al.*, 2009; Zouboulis, 2004). Components of the WNT/ $\beta$ -catenin signaling pathway may therefore serve as targets of cancer treatments (Easwaran *et al.*, 1999; Luu, 2004; Widelitz 2004). Our 3 preliminary studies indicate that RA does indeed interact with WNT signaling in the hair follicle, and inhibition of RA increases WNT signaling in the sebaceous gland. Further studies may help us better understand the quantity of RA needed to regulate WNT signaling in the hair follicle, thus preventing cancer and allowing for hair follicle formation. These studies should be conducted with larger numbers of RARE reporter mice and higher doses of citral in order to determine the specific interactions between RA and WNT signaling in hair follicles.

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